JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2003, p. 5517–5524 Vol. 41, No. 12
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.12.5517–5524.2003

Loop-Mediated Isothermal Amplification for Detection of African Trypanosomes

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Received 25 March 2003/Returned for modification 6 May 2003/Accepted 12 September 2003

While PCR is a method of choice for the detection of African trypanosomes in both humans and animals, the expense of this method negates its use as a diagnostic method for the detection of endemic trypanosomiasis in African countries. The loop-mediated isothermal amplification (LAMP) reaction is a method that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions with only simple incubators. An added advantage of LAMP over PCR-based methods is that DNA amplification can be monitored spectrophotometrically and/or with the naked eye without the use of dyes. Here we report our conditions for a highly sensitive, specific, and easy diagnostic assay based on LAMP technology for the detection of parasites in the *Trypanosoma brucei* group (including *T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, and *T. evansi*) and *T. congolense*. We show that the sensitivity of the LAMP-based method for detection of trypanosomes in vitro is up to 100 times higher than that of PCR-based methods. In vivo studies in mice infected with human-infective *T. brucei gambiense* further highlight the potential clinical importance of LAMP as a diagnostic tool for the identification of African trypanosomiasis.

African trypanosomes are medically and agriculturally important protozoan parasites that cause sleeping sickness in humans and nagana in cattle. Since African trypanosomosis is fatal if left untreated or misdiagnosed, specific and sensitive detection methods are required if early and life-saving treatment for the disease is to be initiated. PCR has evolved as one of the most specific and sensitive methods for the diagnosis of infectious diseases, and many applications of PCR for detecting pathogenic microorganisms have been reported (7, 8, 10, 11, 17, 26). However, problems of reproducibility of PCR diagnosis of human African trypanosomosis, especially on samples from serologically positive but apparently aparasitemic cases, are also reported (26). Moreover, it has been pointed out that *Taq* DNA polymerase is easily inactivated by tissue- and blood-derived inhibitors, such as myoglobin, heme-blood protein complex, and immunoglobulin G (1, 2, 5, 16). These findings appear to indicate the difficulty in optimizing the reaction conditions in PCR.

Recently, a powerful application of PCR, termed real-time PCR, was developed, and applications of a real-time PCR to protozoan parasites have been reported (6, 9, 19, 21). Rapid quantitation and detection of *Trypanosoma cruzi* and *Leishmania* infections by real-time PCR have been reported, and their application for diagnosis appear to be possible (9, 21). However, in spite of excellent specificity and sensitivity of PCR and real-time PCR, these methods are not commonly used in the diagnosis of African trypanosomosis. The reason for this is based more on economics and practicality than need, for in developing nations where African trypanosomosis is endemic, the automated thermal cyclers and/or real-time quantitative PCR thermal cyclers required for the methods are often not affordable and might work erratically at high ambient temperatures and humidity and/or in dusty environments. Therefore, the identification of African trypanosomes in clinical samples still relies heavily on relatively insensitive microscopic observation of blood smears and cerebrospinal fluid. Therefore, cost-effective, simple, and rapid DNA amplification methods for the diagnosis of early and advanced African trypanosomosis are clearly needed.

Loop-mediated isothermal amplification of DNA (LAMP) may provide one answer. LAMP, a method recently developed by Notomi et al. (23), relies on autocycling strand displacement DNA synthesis by a *Bst* DNA polymerase. LAMP requires two specially designed inner and two outer primers (Fig. 1A); as such, LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. Since the LAMP reaction is done under isothermal conditions (63 to 65°C), simple incubators, such as a water bath or block heater, are sufficient for the DNA amplification. Moreover, LAMP synthesizes 10 to 20 μg of target DNA within 30 to 60 min, and the LAMP reaction appears to be limited only by amount of deoxynucleoside triphosphates and primers (12, 23). In the process, a large amount of pyrophosphate ion is produced, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by-product (20). This phenomenon allows easy and rapid visual identification that the target DNA was amplified by LAMP. Therefore, LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of an infectious disease both in well-equipped laboratories and in field situations.

In this study, LAMP primer sets specific for either the *T.*

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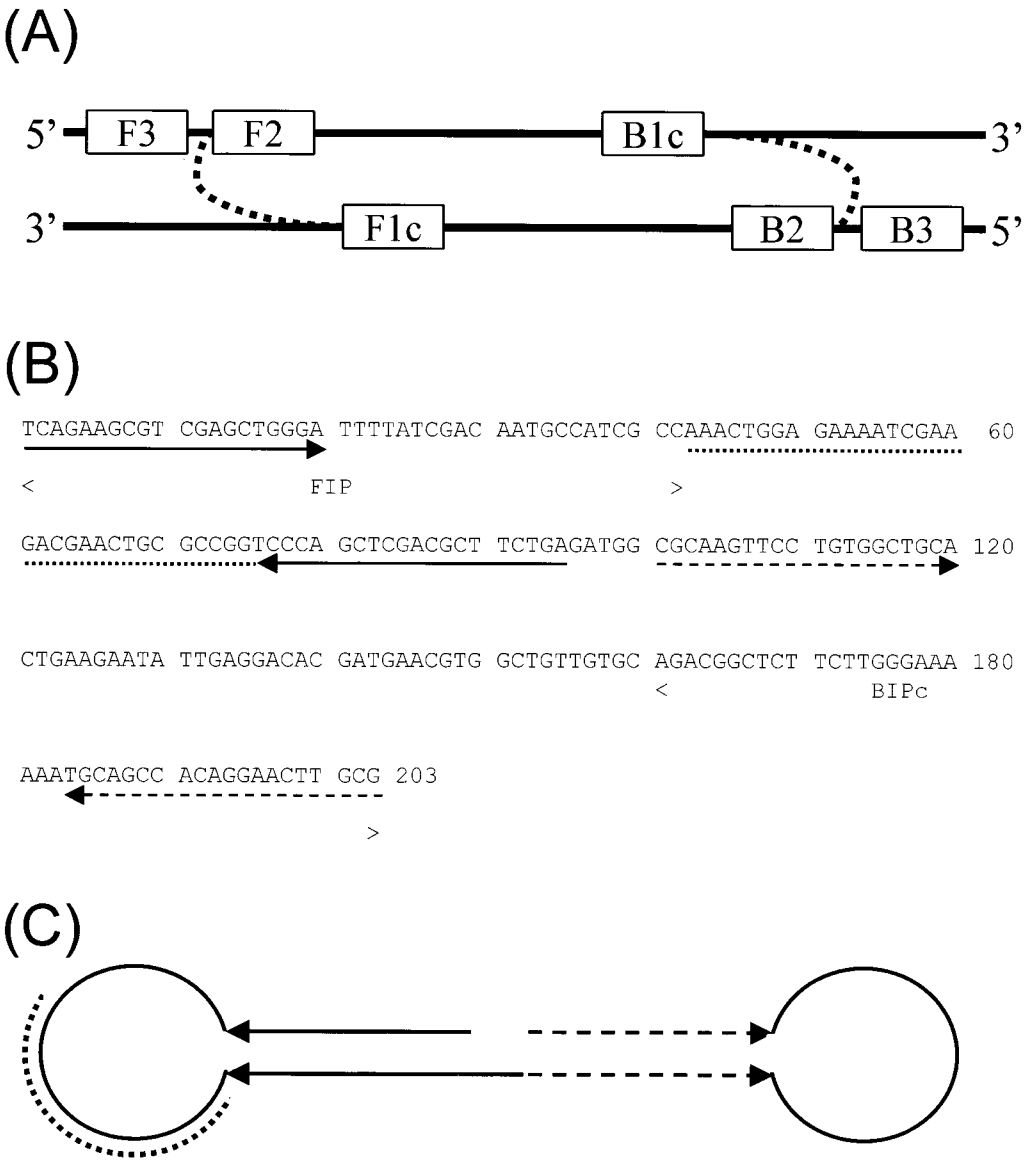


FIG. 1. (A) Schematic presentation of a double-stranded target DNA (solid lines) and LAMP inner (FIP and BIP) and outer (F3 and B3) primer pairs (open boxes). The FIP (BIP) primer consists of F1c (or B1c), a TTTT spacer (dotted line), and F2 (B2). (B) Nucleic acid sequence of minimum *PFR A*-specific LAMP (primer set A1, see Table 1) reaction unit. Two inverted repeats are indicated by solid arrows and dotted arrows. FIP and BIP primers are indicated below the sequence as < FIP > and < BIP >, respectively. A probe used for Southern blot analysis of LAMP products is designed to hybridize the region indicated by dotted line. (C) Schematic presentation of the single-stranded minimum LAMP reaction unit. Inverted repeats at both ends (solid and dotted lines) of the fragment form stem-loop structures. A probe used for Southern blot analysis of LAMP products is designed to hybridize the region indicated by the dotted line.

brucei group (*T. brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesiense*, and *T. evansi*) or *T. congolense* were designed. A LAMP reaction specific for the *T. brucei* group was evaluated for specificity and sensitivity in vitro as well as in vivo, and the results were compared with both microscopic observations and classic PCR.

MATERIALS AND METHODS

Cells. The protozoan parasites and the mammalian cells used for our studies were *T. brucei brucei* GUTat3.1, *T. brucei gambiense* IL-3253, *T. brucei rhodesiense* IL-1501 and IL-2343, *T. evansi* Tansui, *T. congolense* IL-3000, *T. cruzi* Tulahuen, *Theileria orientalis*, *Babesia bigemina*, *B. bovis*, *B. caballi*, *B. equi*, *Toxoplasma gondii* RH, *Neospora caninum*, NIH 3T3 (ATCC CRL-1658), HCT-8 (ATCC CCL-244), MDBK (ATCC CCL-22), and Vero (ATCC CCL-81) cells. With the exceptions of *T. brucei gambiense* IL-3253 and *Theileria orientalis*, all parasites and cells were maintained in vitro (4, 14). *Trypanosoma brucei gambiense* IL-3253 was propagated in SCID mice (15) and purified from infected blood by DE52 anion-exchange column chromatography (18). *Theileria orientalis* was obtained from infected cattle blood.

DNA extraction. Total DNA was extracted from parasites and mammalian cells by published methods (25). Briefly, lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per ml) was added to the samples, followed by overnight incubation at 55°C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. The purified DNA was dissolved in 100 μl of sterilized distilled water.

TABLE 1. LAMP primers

Target gene	Set	Specificity[a]	Sequence
PFRA	A1	FIP	5′-TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC-3′
		F3	5′-TCACAACAAGACTCGCACG-3′
		BIP	5′-CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT-3′
		B3	5′-GGGCTTTGATCTGCTCCTC-3′
	A2	FIP	5′-ATGGCGTGACTTGACGGCACTTTTCTGCATGGGTATGCTGGAG-3′
		F3	5′-TGTGTACAACTGCGACCTTG-3′
		BIP	5′-TGAGTTGTCTGACCTTCGGCTGTTTTGTTTTGTACAGGCGACGGA-3′
		B3	5′-GTACACAAGCTGGCCAAGA-3′
P0	P01	FIP	5′-ATCCGTCGCCTTGCTGTCCTTTTTATGGGGAAGAAGACGCTTCA-3′
		F3	5′-CGTGGTAAGGGTGAATTGGT-3′
		BIP	5′-CAAGCAGCTGCTGTGCGGTATTTTTGATCTCCGTAACGTCCTCG-3′
		B3	5′-GTGTCCGTCCAACACCTTC-3′
	P02	FIP	5′-ATCATGTGCGGGAGCGTAGCTTTTAGGGCATCAGCAACATCAG-3′
		F3	5′-CGACGTTGTGGAGAAGTACC-3′
		BIP	5′-GCATTTAAGACCCTCCTCGGGGTTTTTGTCGCAGGTTCTTACCGT-3′
		B3	5′-AGCTTGCCTTCCAGAGCA-3′

[a] FIP, forward inner primer; F3, forward outer primer; BIP, back inner primer; B3, back primer.

When infected blood was used, total trypanosome (i.e., *T. brucei gambiense* IL-3253) DNA was extracted as follows. First, the infected blood was blotted and dried on filter paper (FTA card; Whatman, United Kingdom). The blotted blood was cut out with a 2-mm hole puncher (2.0-mm Harris Micro Punch; Whatman, United Kingdom). A portion of the blotted blood was then washed three times with 200 μl of FTA purification reagent (Whatman, United Kingdom) and twice in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The washed piece of filter paper was used as the source of template DNA for both LAMP and PCR.

Cloning and sequence determination of LAMP products. LAMP products were diluted appropriately with distilled water and amplified by PCR with primers that bind to the F2 region (5′-ATC GAC AAT GCC ATC GCC-3′) and to complementally strand of B1c region (5′-TTC CCA AGA AGA GCC GTC T-3′) shown in Fig. 1A. The PCR product was cloned to pT7Blue-T vector (Novagen Inc.) with Takara ligation kit version 2 (Takara Bio Inc.). The nucleic acid sequence was determined with the BigDye terminator cycle sequencing kit (Applied Biosystems Japan Ltd.).

Oligonucleotide primers. The LAMP reaction needs four oligonucleotide primers: forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (Fig. 1A) (23). All primer sequences were designed with the software program PrimerExplorer V1 (Fujitsu, Japan). Briefly, the design of the two outer primers, F3 and B3, is the same as that of regular PCR primers, while the design of the two inner primers, FIP and BIP, is different from that of PCR. The inner primers bind both sense and antisense strands of target DNA, and two binding regions within the inner primer (F2 and F1c, or B2 and B1c) are connected by TTTT spacer (Fig. 1A and Table 1). Two sets of 4 primers, named A1 and A2, were designed to hybridize to the gene encoding the paraflagella rod protein A (*PFR A*; GenBank accession number X14819) of *T. brucei*. Two other primer sets, named P01 and P02, were designed to hybridize to the gene for the ribosomal P0 subunit protein (*P0*, GenBank accession number AB056702) of *T. congolense*. For the PCRs, the outer primers (B3 and F3) were used as the PCR primer pair. All primer sequences are listed in Table 1.

LAMP reaction. LAMP was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Japan). Briefly, the LAMP reaction mixture (25 μl) contained template DNA, 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 8 U of *Bst* DNA polymerase large fragment (New England Biolabs Inc.), 1.4 mM deoxynucleoside triphosphates, 0.8 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, and 0.1% Tween 20. As a negative control, template DNA was omitted from the reaction. The reaction mixture was incubated at 65°C for 1 h and heated at 80°C for 2 min to terminate the reaction. The mechanism of LAMP reaction was well explained by Notomi et al. (23). In addition, Hafner et al. reported that the isothermal in vitro amplification and multimerization of linear DNA targets (linear target isothermal multimerization and amplification) with two primers and *Bst* DNA polymerase (12).

The LAMP reaction relies mainly on autocycling strand displacement DNA synthesis that is similar to the cascade rolling-circle amplification reported by Hafner et al. (12). However, there is a possibility that linear target isothermal multimerization and amplification also occurs during the LAMP reaction. The minimum LAMP reaction unit consists of two inner primers (FIP and BIP) and target DNA, as shown in Fig. 1B. Each inner primer contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA and form stem-loop structures at both ends of the minimum LAMP reaction unit (Fig. 1C). These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction. The LAMP products were electrophoresed in a 1.5% Tris–acetic acid–EDTA (TAE) agarose gel. Gels were stained with ethidium bromide solution (1 μg/ml).

PCR. PCRs were carried out under standard and enhanced conditions. Standard conditions of PCR (designated PCR 1) are as follows. The PCR mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each of the four deoxynucleoside triphosphates, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd., Japan). The reaction mixtures were incubated in a programmable heating block (Whatman Biometra GmbH, Germany) at 94°C for 10 min as an initial denaturation step and then subjected to 30 cycles consisting of 45 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a terminal elongation for 7 min at 72°C. On the other hand, enhanced PCR (designated PCR 2) was performed as follows. The PCR mixture (50 μl) contained 10 μl of 5x Ampdirect-D (Shimadzu Biotech Co., Japan), 2 mM each of the deoxynucleoside triphosphates, 5 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Takara Bio Inc., Japan). The reaction program is the same as that of PCR 1 except thermal cycling was repeated 40 times. Ampdirect-D is a reagent capable of effectively neutralizing the substances that inhibit DNA amplification (22). The PCR products were electrophoresed in a 1% TAE agarose gel and the gels were stained with ethidium bromide solution (1 μg/ml).

Southern blot analysis. Each LAMP product (5 μl) was electrophoresed in a 1.5% TAE agarose gel and transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech Ltd., United Kingdom) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was probed under stringent conditions with 5′-biotin-labeled synthetic oligonucleotide probe (*PFR A*: 5′-biotin-AAA CTG GAG AAA ATC GAA GAC GAA CTG CGC CGG-3′, *P0*: 5′-biotin-TCA GAC AAG CTG TTT CAC CAG ACC TGC GCC GA-3′). The probes do not hybridize to either the inner (FIP and BIP) or outer (F3 and B3) primer binding regions, as shown in Fig. 1 (B and C) in order to confirm target-specific LAMP reactions. Streptavidin-alkaline phosphatase (Roche Diagnostics Co., Germany) and CDP-Star detection reagent (Amersham Pharmacia Biotech Ltd., United Kingdom) were used for detection.

Chronically infected mice and blood samples. Five 16 week-old female ICR mice (CLEA Japan, Inc.) were infected intraperitoneally with 10^4 *T. brucei gambiense* IL-3253 bloodstream forms. Every other day for 30 days, approximately 30 μl of blood was collected into hematocrit tubes from the tail vein. Then

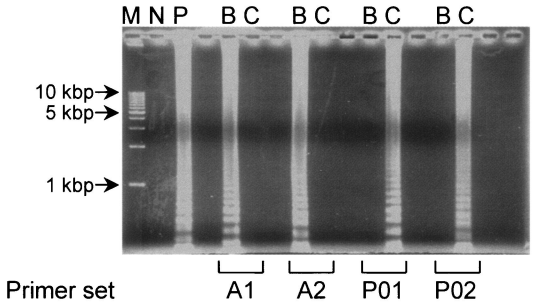


FIG. 2. LAMP reactions for *T. brucei* and *T. congolense*. Four sets of primers were designed to hybridize to the gene encoding *T. brucei* PFR A (A1 and A2) and *T. congolense* ribosomal subunit protein P0 (P01 and P02). The LAMP products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Template DNAs were obtained from *T. brucei* GUTat 3.1 (B) and *T. congolense* IL-3000 (C). Size markers (1-kbp ladder) were electrophoresed in lane M, and their sizes are indicated on the left. Lanes N and P, negative and positive reaction controls, respectively.

10 μl of whole blood was centrifuged for 5 min at 10,000 × *g* to obtain the buffy coat. A drop of the buffy coat was placed on a glass slide and examined for motile parasites under a light microscope at 100× magnification. The remaining (20 μl) whole blood was blotted onto filter paper (FTA card, Whatman, United Kingdom) for total DNA preparation. The blood blots were air dried and stored at room temperature until DNA extraction.

RESULTS AND DISCUSSION

Amplification of *PFR A* and *P0* by LAMP. Two sets of primers were designed for the *T. brucei PFR A* and *T. congolense P0* amplifications. To examine whether these sets of primers were able to amplify their target genes, LAMP reactions were conducted and analyzed by agarose gel electrophoresis. All of the primer sets amplified their target sequences in *PFR A* of *T. brucei* or *P0* of *T. congolense*, and the LAMP products appeared as a ladder of multiple bands (Fig. 2). This amplification pattern is characteristic of the LAMP reaction and indicates that stem-loop DNAs with inverted repeats of the target sequence were produced (23).

Sensitivity and sequence specificity of LAMP. Since the outer primer pair, designated F3 and B3, can also be used for PCR, the same target gene was amplified from serially diluted total trypanosome DNA by both LAMP and standard PCR (PCR 1, see Materials and Methods), and the sensitivities of the two methods were compared.

Figure 3A shows the results of LAMP and PCR 1 with primer sets A1 and P01. LAMP with primer set A1 successfully amplified *T. brucei PFR A* from 1 pg of total DNA, whereas the detection limit with PCR1 with primers A1-F3 and A1-B3 was 100 pg. However, LAMP with primer set P01 required 1 ng of total *T. congolense* DNA for detection, and its sensitivity was 10 times less than that of PCR 1 with primers P01-F3 and P01-B3. Likewise, the detection limits of LAMP with primer sets A2 and P02 were the same as PCR 1 (data not shown). The same agarose gel shown in Fig. 3A was used for a Southern blot, and the result clearly indicated both the LAMP products and the PCR products derived from *T. brucei PFR A* and *T. congolense P0*, respectively (Fig. 3B). In this experiment, template DNA was isolated from DE52 column-purified trypanosomes, and no or minimum contamination of blood components that contain several *Taq* DNA polymerase inhibitors (1, 2, 16) was expected. Therefore, we consider that a comparison between the LAMP and standard PCR instead of enhanced PCR is fair.

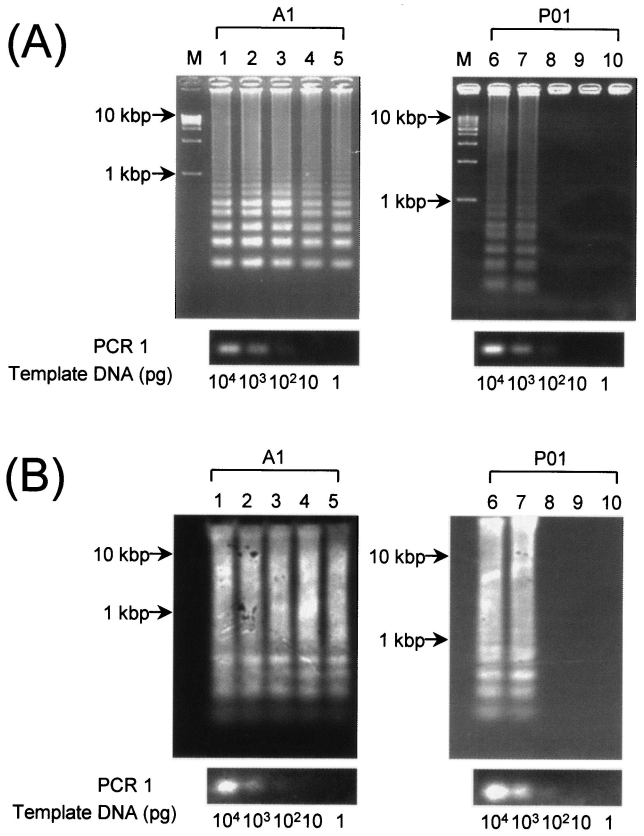
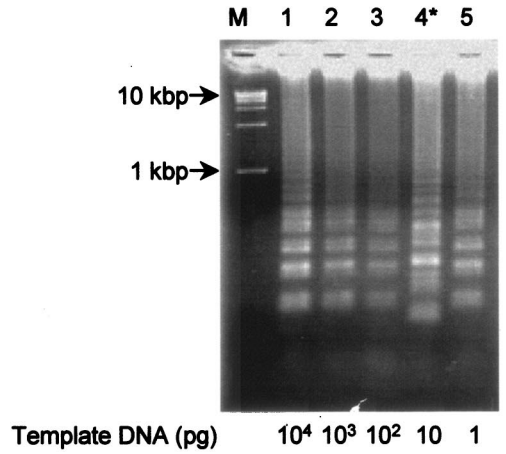


FIG. 3. Comparison of detection sensitivity in LAMP and PCR (A). Total DNAs from *T. brucei* GUTat 3.1 and *T. congolense* IL-3000 were serially diluted from 10 ng to 1 pg and amplified by LAMP and PCR. A1 and P01 are primer sets used in the LAMP reactions. The F3 and B3 primers in each LAMP primer set were used in the PCR. The sizes of the 1-kb size markers in lane M are indicated on the left. (B) Southern blot analyses of the LAMP products. The same LAMP and PCR products shown in A were probed with the synthetic oligonucleotide probes. The probes do not hybridize to either inner (FIP and BIP) or outer (F3 and B3) primer binding regions, as shown in Fig. 1B and C.

A LAMP product of a different band pattern was occasionally observed in *PFR A*-specific LAMP (primer set A1) (Fig. 4A), and such a LAMP product did not hybridize to the oligonucleotide probe (data not shown). In order to characterize the LAMP product with the different band pattern, a part of the LAMP products was amplified by PCR, and then the PCR product was cloned into pT7Blue-T vector (Novagen Inc.) as described in Materials and Methods. The nucleic acid sequence of the PCR-amplified LAMP product is shown in Fig. 4B (Clone 1). Clone 1 contained only LAMP primer and short *PFR A* sequences ($PFR\ A_{512\text{-}526}$: CTT CTG AGA TGG CGC) (Fig. 4B, clone 1). Although, the order of each primer in clone 1 was not the same as that of a regular LAMP product (Fig. 4B, LAMP), we concluded that the LAMP reaction of different band pattern (Fig. 4A, lane 4*) was not the results of nonspecific amplification but target DNA specific.

It was reported that *Bst* DNA polymerase has two distinct activities, termed linear target isothermal multimerization and

(A)



(B)

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LAMP     TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC********  42
         <                  FIP                    >
Clone 1                          ATCGACAATGCCATCGCCCGCAAGTT  26
                                                  <  BIP

LAMP     **********************************************  42
Clone 1 CCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCTTCACAACAAGACTCG  76
                 BIP                      ><        F3

LAMP     ****AAACTGGAGAAAATCGAAGACGAACTGCGCCGGTCCCAGCTCGACG  88
             <            PFR A466-511                    >
Clone 1 CACG-------------------------------------------------  80
           >

LAMP     CTTCTGAGATGGCGC***********AAGTTCCTGTGGCTGCACTGAAG 126
         <                PFR A512-549                    >
Clone 1 CTTCTGAGATGGCGCAGATCAAAGCCC---------------------- 107
                       <    B3    >

LAMP     AATATTGAGGACACGATGAACGTGGCTGTTGTGCAGACGGCTCTTCTTGG 176
         <                PFR A550-582      ><      BIPc
Clone 1 ----------------------------------AGACGGCTCTTCTTGG 123


LAMP     GAAAAAATGCAGCCACAGGAACTTGCG 203
                 BIPc             >
Clone 1 GAA                        126
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FIG. 4. Characterization of a different amplification pattern of *PFR A*-specific LAMP. (A) Total DNA from *T. brucei* GUTat 3.1 was serially diluted from 10 ng to 1 pg and amplified by *PFR A*-specific LAMP. *PFR A* A1 primer sets were used in the LAMP reactions. The band pattern of lane 4* is different from the others (lanes 1, 2, 3, and 5). The sizes of the 1-kb size markers in lane M are indicated on the left. (B) Comparison of nucleic acid sequences between the regular LAMP product (LAMP) and that of lane 4* (Clone 1). Sequence features are described between the < and > signs. BIPc indicates the complementary strand of the BIP primer. Insertions and deletions found in clone 1 are indicated by asterisks and hyphens, respectively.

amplification and cascade rolling-circle amplification (12). In the same manuscript, target DNA-specific amplification of both linear target isothermal multimerization and amplification and cascade rolling-circle amplification was also proved (12). The mechanism of the loop-mediated isothermal amplification reaction is similar to that of the cascade rolling-circle amplification. An occasional different LAMP amplification pattern appears to be the result of linear target isothermal multimerization and amplification, because LAMP primers and target DNA seem to be randomly multimerized.

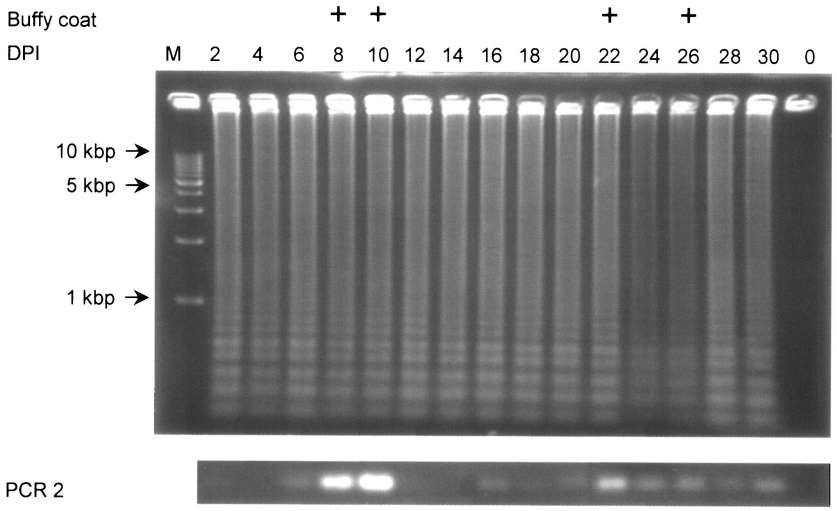

FIG. 5. Sequential analysis of blood samples obtained from mouse 1 infected with *T. brucei gambiense* IL-3253. The samples were examined by microscopic observation of buffy coat samples, *PFR A*-specific LAMP with primer set A1, and *PFR A*-specific PCR with primers F3 and B3 of the A1 primer set. PCRs were performed under enhanced conditions as described in Materials and Methods. Numbers above each lane indicate days postinfection (DPI). + indicates the presence of trypanosomes in buffy coat samples observed by microscopy. The sizes of the markers in lane M are indicated on the left.

Species specificity of LAMP. Since LAMP with primer set A1 showed 100 times higher sensitivity than PCR, further evaluation of the LAMP reaction was carried out. It has been reported that *T. evansi* is evolutionarily closely related to the three subspecies of *T. brucei*, *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense*, and that its genomic DNA is indistinguishable from that of *T. evansi* (3, 13, 24, 27). Therefore, we tested whether LAMP with primer set A1 would give the same positive reactions with 10 ng of template DNA from the *T. brucei* subspecies and *T. evansi*. Total DNA from *T. brucei rhodesiense*, *T. brucei gambiense*, and *T. evansi* was subjected to LAMP, and all showed a positive reaction.

Because the areas of distribution of African trypanosomes and *T. evansi* overlap those of many kinds of protozoan parasites, there is every possibility of mixed infection with trypanosomes and other parasites. Therefore, the specificity of the LAMP was also tested on protozoan parasites such as *Trypanosoma cruzi*, *Theileria orientalis*, *Babesia bigemina*, *B. bovis*, *B. caballi*, *B. equi*, *Toxoplasma gondii*, and *Neospora caninum*. Moreover, genomic DNAs of mammalian hosts, namely, human, monkey, bovine, and murine, were subjected to the LAMP in order to examine its specificity. The protozoan parasites and mammalian cell DNAs listed above were all negative in the *PFR A*-specific LAMP (primer set A1). Thus, the results indicate that the LAMP reaction can detect both *T. brucei* and *T. evansi* with high sensitivity and specificity.

A LAMP reaction requires four primers that recognize six different sequences on a target sequence (Fig. 1A) (23). At the first step of a LAMP reaction, *Bst* polymerase synthesizes new DNA strands from the F3 and B3 primers. This reaction is the same as PCR and requires sequence homology between a primer and a target. At the next step, the newly synthesized strands should be recognized by inner primers (FIP and BIP) in order to start loop-mediated autocycling amplification. Therefore, the target sequence specificity of a LAMP reaction appears to be higher than that of PCR (23).

Detection of *T. brucei gambiense* DNA from blood samples. The LAMP studies reported above were conducted with purified template DNA. However, to diagnose trypanosomosis in the field, the method has to be able to detect parasites in whole blood or cerebrospinal fluid, the most common clinical materials for examination. Therefore, five mice were injected with *T. brucei gambiense* IL-3253, which has a low virulence in mice. All five mice became infected, and every other day blood samples were collected from the tail vein, and the parasitemia of each mouse was examined by microscopic observation of thin smears obtained from the buffy coat.

To simplify DNA extraction procedures, we used commercially available reagents, the FTA card and FTA reagent (Whatman, United Kingdom). The FTA card is a chemically treated filter paper that allows the rapid isolation of pure DNA. When samples are applied to the FTA card, cell lysis occurs and high-molecular-weight DNA is immobilized within the matrix. Thus, a small piece of the FTA card can serve as the template DNA source after washing several times with FTA reagent and Tris-EDTA buffer. In the case of mouse 1 (Fig. 5), trypanosomes were detected by microscopic observation at 8, 10, 22, and 26 days postinfection, and trypanosome DNA was detected by LAMP reaction at all days postinfection. We also tried to detect trypanosome DNA by PCR. At first, PCR was performed under standard conditions (designated PCR 1). However, PCR 1 could amplify trypanosome DNA only at 10 days postinfection (data not shown). The same DNA samples from mouse 1 were subjected to PCR 2. As a result, trypanosome DNA was first detected at 6 days postinfection, and band intensities of PCR 2 products increased from 6 days postinfection to 10 days postinfection (Fig. 5). A change in the magnitude of band intensity in the PCR 2 appears to correspond to the result of microscopy.

The results for other mice are shown in Table 2. These results clearly indicate the extremely high sensitivity of the LAMP reaction. However, we occasionally observed false-pos-

TABLE 2. Sequential analysis of blood samples from mice infected with *T. brucei gambiense* IL3253

Mouse no.	Method	Result[a] on day postinfection															
		0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
2	Buffy coat	−	−	+	−	−	−	−	+	−	−	+	+	−	−	−	+
	PCR 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PCR 2	−	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+
	LAMP	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Buffy coat	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PCR 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PCR 2	−	−	−	−	−	+	−	+	+	−	+	+	+	+	+	+
	LAMP	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
4	Buffy coat	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PCR 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PCR 2	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LAMP	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Buffy coat	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
	PCR 1	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
	PCR 2	−	+	+	+	−	+	+	+	+	−	+	−	+	+	+	+
	LAMP	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+

[a] + and − indicate the presence and absence of trypanosomes, respectively.

itive LAMP reactions in negative controls (data not shown). The false-positive reactions were probably due to parasite contamination and/or amplicon cross contamination. Careful precautions against such cross-contamination must be taken during sample collections and preparations for LAMP. Furthermore, the sensitivity of PCR 2 (Fig. 5 and Table 2) was significantly higher than that of PCR 1. Thus, PCR conditions must carefully optimized. The marginal template DNA concentration for the positive reaction in PCR 1 was 100 pg (Fig. 3). With PCR based on the Te664 DNA fragment and ethidium bromide staining, Ventura et al. (23) reported that ≈10 pg of total *T. evansi* DNA represents ≈25 cells (27). Therefore, marginal detection of our PCR for *PFR A* is about ≈250 cells.

Because we wished to compare the sensitivity of the LAMP and PCR methods targeted to the same gene, we intentionally used PCR primers A1-F3 and A1-B3. It has been shown that microscopy can detect <1,000 parasites in buffy coat material obtained from 1 ml of trypanosome-infected blood (28). The template DNA sources for our PCR experiments were obtained from less than 5 μl of blood blotted as a 2-mm diameter onto filter paper. In a sample containing ≈1,000 parasites/ml, we could expect 5 μl of blood to contain five parasites on the filter paper, an amount that is within the detection limit of light microscopy. Therefore, we conclude that PCR 1 was less sensitive than microscopy, because PCR 1 required at least 250 parasites on the filter paper for detection. On the other hand, PCR 2 could amplify trypanosome DNA with high sensitivity and showed higher sensitivity than the LAMP at 20 days postinfection for mice 3 and 5. Differences between PCR 1 and PCR 2 are a repeat of thermal cycling and addition of the PCR enhancer termed Ampdirect-D (Shimadzu Biotech Co., Japan). Therefore, the results suggests that the FTA card preparation cannot completely remove blood components that inhibit *Taq* DNA polymerase activity. In fact, it was reported that blood-derived materials such as heme-blood protein complex strongly inhibited *Taq* polymerase activity (1). Thus, we speculate that the lower sensitivity of PCR1 is due to the lower purity of the template DNA, which was extracted with the FTA card.

Compared to PCR, LAMP has the advantages of reaction simplicity and detection sensitivity. LAMP does not require complicated thermal cycling steps; an isothermal reaction for a rather short time (≈1 h) is enough to amplify the target DNA to detectable levels. Another useful feature of LAMP lies in the opportunity for turbidity-based detection of the positive reaction (20). The turbidity of the LAMP reaction mix can be easily judged by the naked eye. In all cases, we could distinguish LAMP-positive samples from negative samples simply by the turbidity of the reaction mixtures (data not shown). Because PCR and other molecular biological techniques are best conducted only in well-equipped laboratories, these methodologies are often impracticable under conditions requiring a field diagnosis. In contrast, the useful characteristics of LAMP that we have described make it possible to use this highly sensitive DNA amplification method in many places, under field conditions and in local clinics and hospitals where cost and environmental restraints prohibiting PCR are otherwise in effect. While we have taken an important first step, further improvements are still needed, i.e., with our current primers, LAMP detects both *T. brucei* and *T. evansi*. Even so, LAMP will still be useful for the initial screening of suspected infection caused by *T. brucei* species and *T. evansi*, important causative agents of trypanosomosis in humans and animals.

ACKNOWLEDGMENTS

We thank John E. Donelson (Department of Biochemistry, University of Iowa) for thoughtful discussions and thank him and J. Stephen Dumler (Department of Microbiology, Johns Hopkins School of Medicine) for critical reading of the manuscript.

This work was supported by a Grant-in-Aid for Scientific Research (no. 13356048) to N.I. from the Japan Society for the Promotion of Science and, in part, by a grant from the National Institutes of Health (1 RO1 AI51464-01) to D.J.G.

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